



Regular Article

Endogenous Expression of Galectins and Tissue Binding Profile of a Galactose Specific Plant Lectin in Cervical Intra Epithelial Neoplasia: It's Significance to Assess the Malignant Potential

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ABSTRACT: Galectins are a family of low molecular weight β -galactoside binding proteins with functions in cell growth, cell activation, cell-cell, cell-matrix adhesion including binding to carcinoembryonic antigen, laminin and metalloprotenase. Higher endogenous expression of Galectin-3 (Gal3) and Galectin-1 (Gal-1) has been found in malignancies. Several other galactose specific lectin binding patterns have been reported to differentiate dysplastic epithelium from normal epithelium. Galectin expression studies in cervical epithelium are comparatively less. We analyzed the endogenous expression of Gal-1, Gal-3 and the binding profile of a plant lectin with identical sugar specificity in cervical intra epithelial neoplasia (CIN) to evaluate its significance as a marker to assess the malignant potential of CIN. Sections from 66 archival samples of colposcopic biopsy were analyzed using the standard immunohistochemical method using polyclonal anti-galectin antibodies and HRP conjugated snake gourd lectin using DAB as the chromogen. The expression pattern was assessed semi quantitatively and analysed statistically to see the significance as a predictor of the malignant potential of CIN. Galectin expression was observed in cytoplasm and cell membrane. Neither the galectins nor the plant lectin seemed to have any role in predicting malignant potential if cervical intra epithelial lesion. As these lectins express more in differentiated epithelium this can be suggested as markers of differentiation which may have some significance in tumor pathology.

Key words: Galectins, Snake gourd lectin binding, Preselection, CIN

Introduction

Cancer of the uterine cervix is the second most common cancers among women worldwide. It accounts for 15% of all cancers diagnosed among women. Nearly 20% of these cancers occur in developed countries and 80% in developing countries. In India, according to the data available from population based cancer registries at Bangalore, Madras and Bombay, the crude incidence rates are 39.7, 46.5 and 15.4 per 100,000 females respectively [1]. Invasive cervical carcinoma is preceded by a spectrum of heterogeneous epithelial changes called pre-cancerous lesions or dysplasia or cervical intra epithelial neoplasia (CIN) [2]. These pre-invasive lesions usually develop through several grades namely mild, moderate and severe dysplasia or CIN I, II and III or SIL (Squamous Intra epithelial Lesion) low and high grades, which may lead to invasive cancer over a period of time, if left untreated. The rate of progression of these lesions to cancer has been reported to vary from 4.4% to 65% depending upon the severity of the lesion. Studies have demonstrated progress from CIS (Carcinoma In situ) to invasive carcinoma to range from 25-70% [2]. In the British Columbia cohort study, for cohort I, 61% of the cases of CIS were estimated to be regressed during the age of 40-60 years. For cohort II, the regression rates were 70% over the age of 25-44 years and 77% over the age of 20-39 years. Murthy has found a relative risk of developing CIS in various grades of dysplasia to be 5.1, 3.73 and 114.4 respectively for mild, moderate and severe dysplasia [3]. Among the 1107 dysplasia patients followed up, 64 (5.8%) revealed malignancy at the first follow up within three months of initial recruitment, while another 79% developed malignancy during

subsequent years. It was 0.66 for mild, 5.0 for moderate and 16.1 per 100 women years for severe dysplasia [3]. The role of Human Papilloma Virus in the development of early CIN has been established. HPV type 16, 18 and others has been reported to involve in the etiology of at least 90% of the Indian cervical cancers. It has been reported that HPV infection alone cannot be considered as predisposing factor for CIN and a considerable number of CIN cases will revert to normal or remain as such without progressing to invasive carcinoma [2]. If biological markers can accurately predict the CIN lesions which have more chance to progress to higher up lesions, treatment and further cytological follow up can be limited to them alone instead of managing all the dysplasias. Interest has therefore been focused to identify markers to identify high risk CIN cases which have the potential to progress to invasive carcinoma and that can be used as a pre-selection criterion for further management. Studies on oral premalignant lesions exhibiting epithelial dysplasia have shown changes in the glycosylation pattern, where as such changes are only occasionally seen in lesions without dysplasia [5,6]. Lectins are reported to be useful in identifying these changes in the glycosylation pattern. The present study analyzed the pattern of expression of endogenous lectins, galectin-1, galectin-3 and a galactose specific plant lectin, Snake gourd lectin (SGL) binding pattern in normal, different grades of CIN and malignant tissues of cervix to see whether the SGL binding pattern or the galectin expression show any significant difference between different grades of dysplasia and malignancy so that galectose specific ligand study can be used to preselect high risk cervical intra epithelial lesions.

Materials and Methods

Lectin from the seeds of snake gourd was isolated, purified and conjugated to horseradish peroxidase type IV according to the technique described elsewhere [6,7]. In short, the lectin isolated from the seeds by ammonium sulphate fractionation was purified by affinity chromatography on cross-linked guar gum. The molecular weight of the lectin was found out by gel filtration on sephadex G-100 (Sigma chemical company). The purity of the lectin was confirmed on electrophoretic analysis. The purified lectin was conjugated to horseradish peroxidase type IV. Polyclonal anti-galectin antibodies of Gal-1 and Gal-3 were purchased from Novocastra. Sections from archival blocks of colposcopy directed punch biopsy specimens were used for this study. Normal cervical tissues were obtained from hysterectomy specimens. A total of 66 samples were analyzed, which includes normal (12), CIN I (11), CIN II (10), CIN, III (10), Invasive squamous cell carcinoma (16) and adenocarcinoma (7) cases. Representative sections were selected by correlating with the original H&E sections used for the initial diagnosis. Standard immunohistochemical method was followed using DAB as the chromogen. The sections were incubated with primary antibodies / SGL at 4°C for one hour. Anti galectin antibodies were used in a concentration of 1:100 dilutions and the SGL was used in a concentration of 0.06 μ /ml. Negative controls were included for each batch. The extent of specifically bound markers was stained in 30% 3'-3'-diaminobenzidine containing hydrogen peroxide for 3-5 minutes. The sections were then counterstained with haematoxyline. The intensity of the lectin binding was assessed on

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a 0-3+ scale in different levels of maturation of the cervical epithelium in the different grades of cervical intra epithelial neoplasia and in malignancies. The percentage of cells with each grades of lectin binding patterns were assessed. The mean score along with standard deviation, standard error mean and 95% confidence interval for each levels of epithelium in normal and in different grades of CIN were statistically analyzed giving specific values 1,2 and 3 for +, ++, and +++ respectively. Malignancies were exempted from the analysis owing to the uniform pattern of expression.

Results

Galectin-3 and galectin-1 expressions were observed in all of the normal, all grades of CIN and in malignancies. The expression pattern of galectins as mean score for each levels of epithelium is given in table I. The expression pattern for both the galectins were found to vary in different maturation levels of the epithelium (Fig I). The most mature superficial layers of all the samples showed expression of both of the galectins with a mean score of 1.5 for gal-1 and 1.6 for gal-3. Whereas, expression of both the gal-3 as well as gal-1 was found to decrease towards the deeper immature layers. The mean value for gal-3 and for gal-1 in the mid zone were 0.67 and 0.83, whereas it was 0.50 and 0.67 in the basal zone. For CIN I

as well as CIN II, the pattern of expression of both the galectins was more or less similar to that of normal epithelium (Fig II). Higher expressions were confined to the differentiated layers only. The mean score for gal-3 and gal-1 were 1.55, 1.30 & 1.09, 1.50 with 95% confidence intervals of 0.99 - 2.10, 0.95 - 1.65 & 0.62 - 1.5, 1.12 - 1.88 for superficial layer in CIN I & II whereas, it was 1 & 0.91, 1.30 with 95% confidence interval of 0.42 - 1.52, 0.66 - 1.34 and 0.35 - 1.47, 0.95 - 1.65 for mid zone. The basal zones showed the mean values as 0.45, 0.50 and 0.73, 0.70 respectively. No significant difference was observed between normal and dysplastic epithelium except for severe dysplasia, which showed a mean value of 1 and 0.9 for gal-3 and gal-1 for the basal layer also. In malignancies, the expressions were higher for both of the galectins and was found to increase with differentiation uniformly in all cases of Squamous cell carcinomas (Fig III). As no difference was observed in the expression pattern between individual samples of squamous cell carcinomas, malignancies were exempted from statistical analysis. The adenocarcinomas also showed uniform expression pattern for both the galectins. The expression of both gal-3 as well as gal-1 were diffuse, uniform and cytoplasmic. No nuclear expressions were observed. Normal endocervical cells showed apical luminal expression for both the galectins (Fig I). In adenocarcinomas also the expression was luminal (Fig III). Moderate expressions were observed for koilocytes also.

Table I Univariate analysis of Galectin expression pattern in Cervical Epithelium

	Mean		Standard deviation		Standard error mean		95 %CI			
	Gal-3	Gal-1	Gal-3	Gal-1	Gal-3	Gal-1	Gal-3	Gal-1	Gal-3	Gal-1
<i>Normal</i>										
Super	1.67	1.5	0.267	0.55	0.21	0.22	1.22	2.21	0.93	2.07
mid	0.67	0.83	0.52	0.41	0.21	0.17	0.12	1.21	0.40	1.26
basal	0.50	0.67	1.22	0.52	0.5	0.21	0.79	1.79	0.12	1.21
<i>Sq. met</i>										
Super	1.17	1.5	0.41	0.84	0.17	0.34	0.74	1.60	0.62	2.38
mid	0.83	1.17	0.41	0.97	0.17	0.40	0.40	1.26	0.13	2.20
basal	0.83	1	1.17	1.10	0.48	0.45	0.34	2.06	-0.15	1.21
<i>CIN 1</i>										
Super	1.55	1.09	0.82	0.70	0.25	0.21	0.99	2.10	0.62	1.56
mid	1	0.91	0.77	0.83	0.23	0.25	0.42	1.52	0.35	1.47
basal	0.45	0.73	0.873	1.19	0.28	0.36	-0.7	1.18	-7.18	1.53
<i>CIN 2</i>										
Super	1.30	1.50	0.48	0.53	0.15	0.17	0.95	1.65	1.12	1.88
mid	1	1.30	0.22	0.48	0.15	0.15	0.66	1.34	0.95	1.65
basal	0.50	0.70	0.53	0.67	0.17	0.21	-1.7	1.08	0.22	1.18
<i>CIN3</i>										
Super	1.20	1.30	0.42	0.67	0.13	0.21	0.90	1.50	0.82	1.78
mid	0.1	0.10	0.32	0.32	1	1	-1.3	0.33	-0.13	0.33
basal	1	0.90	0.77	0.83	0.23	0.25	0.42	1.52	0.35	1.47

Table II Univariate analysis of SGL binding pattern in cervical epithelium

	Mean		Standard deviation		Standard error mean		95% CI	
<i>Normal</i>								
Superficial	1.28		0.41		0.20		0.74	1.50
Mid	0.57		0.52		0.21		0.12	1.21
Basal	0.50		0.50		0.20		0.10	1.20
<i>Sq. Metaplasia</i>								
Superficial	1.1		0.41		0.17		0.74	1.60
Mid	0.67		0.52		0.21		0.12	1.21
Basal	0.50		0.55		0.22		-7.48	1.07
<i>CIN 1</i>								
Superficial	1.09		0.54		0.16		0.73	1.45
Mid	1		0.45		0.13		0.70	1.30
Basal	0.64		0.50		0.15		0.30	0.98
<i>CIN 2</i>								
Superficial	1.20		0.42		0.13		0.90	1.50
Mid	0.70		0.67		0.21		0.22	1.18
Basal	0.68		0.47		0.19		0.32	0.93
<i>CIN3</i>								
Superficial	1.10		0.57		0.18		0.69	1.51
Mid	0.60		0.70		0.22		0.98	1.10
Basal	1		0.67		0.21		0.52	1.48

SGL binding was also similar to the galectin expression pattern. The mean score for each level of tissue layers is given in the table I. The mature superficial layer showed a higher binding pattern with a mean value of 1.28. The binding intensity in the lower layers was comparatively lesser (Fig I). The mean value for the mid zone and the basal zone were 0.57 and 0.50 for normal epithelium. No significant difference in binding pattern was observed between different grades of dysplasias (Fig II). In CIN I, II and III the intensity of SGL binding were higher in differentiated layers with a mean value of 1.09, 1.20 and 1.10 for CIN I, II and III respectively with 95% confidence intervals of 0.73-1.45, 0.90-1.50 and 0.69-1.57 respectively. The binding patterns found to decrease towards

the deeper layers except in CIN III, for which the basal layer also showed a mean value of 1. Similar to galectin expression the binding was mainly cytoplasmic and the pattern was diffuse uniform. In squamous metaplasia also the differentiated layer showed moderate expression of galectins and moderate SGL binding. Similarly the koilocytes also showed SGL binding in moderate grade (Fig III). Unlike the galectins the normal endocervical cells showed diffuse uniform cytoplasmic binding. In squamous cell carcinomas, similar to galectins the SGL binding was higher in differentiated areas. But in adenocarcinomas the binding was different from galectins, as SGL showed uniform binding all over the cytoplasm (Fig III).

Fig I Expression patterns of Gal-3, Gal-1 and SGL binding in normal, metaplastic and endocervical epithelium of the uterine cervix

- Fig A. Higher (++) diffuse cytoplasmic expression of Gal-3 in the superficial layers of Squamous epithelium (40 X)
 B. Higher (++) diffuse cytoplasmic expression of Gal-1 in the superficial layers of Squamous epithelium (40 X)
 C. Higher (++) diffuse cytoplasmic binding of SGL in the superficial layer of Squamous epithelium (40 X)
 D. Higher (++) diffuse cytoplasmic expression of Gal-3 in differentiated layers of Metaplastic epithelium (40 X)
 E. Higher (++) diffuse cytoplasmic expression of Gal-1 in differentiated layers of Metaplastic epithelium (40 X)
 F. Higher (++) diffuse cytoplasmic binding of SGL in differentiated layers of Metaplastic epithelium
 G. Luminal expression of Gal-3 in the normal mucus secreting endocervical cells (40X)
 H. Luminal expression of Gal-1 in the normal mucus secreting endocervical cells (40X)
 I. Uniform diffuse binding of SGL in the normal mucus secreting endocervical cells (40X).

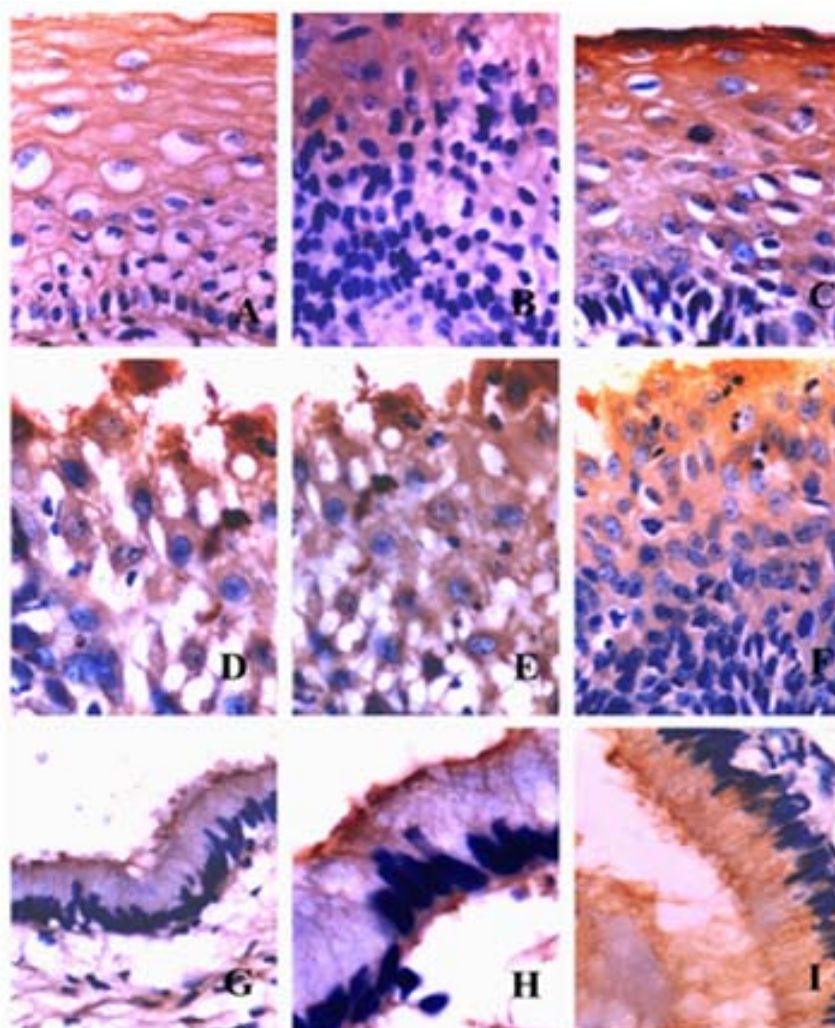


Fig II Expression patterns of Gal-3, Gal-1 and SGL binding in CIN I, CIN II and CIN III.
 Fig A. Higher (++) diffuse cytoplasmic expression of Gal-3 in the differentiated layers in CIN I (40 X)
 B. Higher (++) diffuse cytoplasmic expression of Gal-1 in the differentiated layers in CIN I (40 X)
 C. Higher (++) diffuse cytoplasmic binding of SGL in differentiated layers in CIN I (40 X)

D. Higher (++) diffuse cytoplasmic expression of Gal-3 in the differentiated layers in CIN II (40 X)
 E. Higher (++) diffuse cytoplasmic expression of Gal-1 in the differentiated layers in CIN II (40 X)
 F. Higher (++) diffuse cytoplasmic binding of SGL in the differentiated layers in CIN II
 G. Higher (++) diffuse cytoplasmic expression of Gal-3 in the differentiated layers and mild (+) expression in the basal layer in CIN III (40X)
 H. Higher (++) diffuse cytoplasmic expression of Gal-1 in the differentiated layers and mild (+) expression in the basal layer in CIN III (40X)
 I. Higher (++) diffuse cytoplasmic binding of SGL in the differentiated layers and mild (+) binding in the basal layer in CIN III (40X)

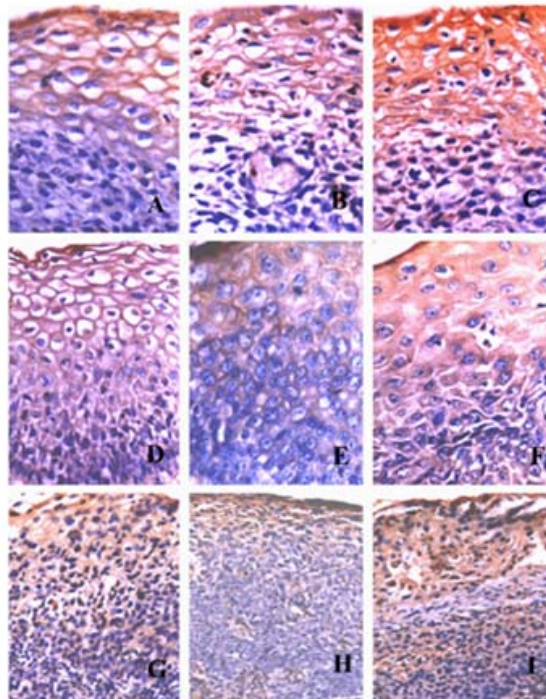
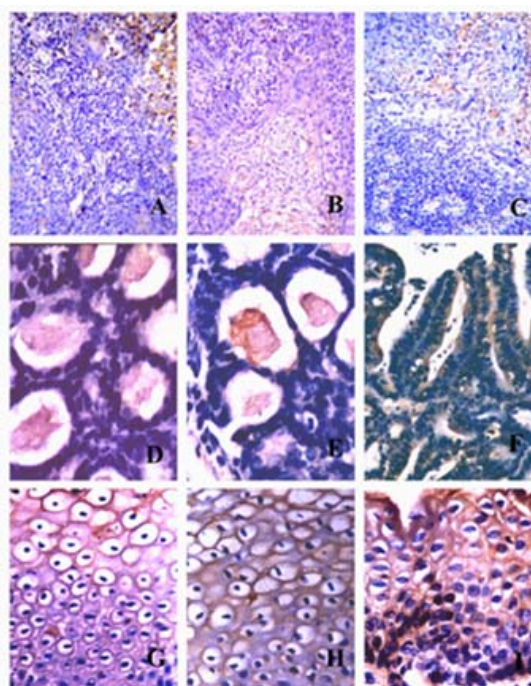


Fig III Expression patterns of Gal-3, Gal-1 and SGL binding in Squamous cell carcinoma, Adenocarcinoma and Koilocytes of the Uterine Cervix.

Fig A. Higher diffuse expression of Gal-3 in the differentiated layers of squamous cell Carcinoma (10X)
 B. Higher diffuse expression of Gal-1 in the differentiated layers of Squamous cell Carcinoma (10X)
 C. Higher diffuse binding of SGL in the differentiated layers of Squamous cell Carcinoma (10X)
 D. Luminal expression of Gal-3 in adenocarcinoma (40 X).
 E. Luminal expression of Gal-1 in adenocarcinoma (40 X).
 F. Diffuse uniform binding of SGL in adenocarcinoma (40X)
 G. Moderate expression of Gal-3 in Koilocytes (40X).
 H. Moderate expression of Gal-1 in Koilocytes (40X)
 I. Moderate binding of SGL in Koilocytes (40X)



Discussion

Cancer of the uterine cervix is characterized by a series of progressive pre-malignant changes ranging from CIN I to CIN III. In developed countries, this disease has been effectively controlled by the introduction of mass screening programmes with the "Pap smear" test. The efficacy of "Pap smear" in detecting cancers and pre cancerous lesions of cervix has been much improved by the introduction of quality control measures [8]. However, in India and in several other developing countries, the infra structure for screening programmes and opportunity for diagnostic work up and treatment may not be sufficient for screening all women. If the high-risk precancerous lesions can be identified from the cytologically detected CIN cases, treatment can be limited to this pre-selected group alone and the disease can be controlled in a much better cost effective way.

There are several reports demonstrating different lectin binding pattern for dysplasias and malignancies of the uterine cervix and oral mucosa [9,10]. JFL has been reported as a good marker of differentiating pre-cancerous lesions in cervical epithelium [11-13]. They have studied the JFL specific glycoconjugates expression during the progression of cervical intra epithelial neoplasia in exfoliated cells. They found that only mild grade of JFL binding in non-neoplastic cells and the binding was reported to increase with increase of cytological atypia. Also they have reported a higher binding pattern associated with squamous differentiation of cells. Normal endocervical cells were reported to show higher lectin binding whereas the metaplastic cells showed a reduction in binding and mild grade of JFL binding had a negative correlation and intense grade of staining had a positive correlation with the stage of the tumor. Naik [14] have studied the binding pattern of a panel of 16 lectins in different grades of vulvar intra epithelial neoplasia (VIN), metastasizing and non-metastasizing squamous carcinomas and have correlated the binding pattern with metastatic potential and other clinical / tumor characteristics. They found no difference in lectin binding pattern between the different histological subtypes of VIN and no consistent difference they observed between metastasizing and non metastasizing primary tumors, also no difference in staining pattern between nodal metastasis and the corresponding primary tumor. They observed no identifiable correlation between lectin binding pattern and subsequent survival or local or regional recurrence. The same groups have analyzed the lectin binding pattern in the vulvar normal squamous epithelium and epithelium adjacent to VIN and squamous cell carcinoma in another study [14]. They found no alteration to lectin binding pattern in normal vulvar epithelium with respect to patient's age, menstrual status, estrogen therapy or history of VIN. Lectins, MPA and LCA were identified as markers of cellular differentiation and maturation. T antigen as shown by the lectin PNA was almost universally present in histologically normal epithelium adjacent to VIN and vulvar tumors, contrasting with the lack of PNA binding in normal vulvar epithelium, a finding suggestive of a local field change surrounding pre-invasive and invasive vulvar lesion, the tumor tissue was observed with a moderate intensity. In the present study, normal and dysplastic epithelium showed moderate binding of SGL in the differentiated superficial layer only. The immature basal layer didn't show any binding in normal as well as in dysplasias. The metaplastic epithelium also exhibited the same binding pattern suggesting the SGL binding pattern as a marker of differentiation rather than the malignant potential of the preneoplastic lesions. In malignancies also the intensity of expression was higher (++) in differentiated areas, whereas the undifferentiated areas showed mild grade (+) of binding only. The difference in SGL binding pattern may be due to the alteration of cell surface glycoconjugates during the malignant transformation which is not recognized in the early neoplastic changes. However, SGL recognizes these changes in high-grade intraepithelial lesions as evident in the SGL binding pattern of the severe dysplastic lesions. As all the severe dysplastic lesions showed the same pattern of binding no pre-selection among these severe dysplasia group could be suggested using SGL binding pattern. Thus, the present study didn't suggest any role for SGL in predicting the malignant potential of CIN and supports the report of Naik in the application of plant lectins in VIN.

Galectins are a family of low molecular weight β -galactoside specific lectins with functions in cell growth, cell activation, cell-cell, cell-matrix adhesion including binding to carcinoembryonic antigen, laminin and metalloproteinase [2]. Gal-3 and Gal-1 have been found to enhance metastatic potential of human tumors by interacting with T antigen in a number of experimental studies [15-19]. Galectin expression studies in cervical epithelium are comparatively less. To our knowledge this is the first report. However galectin expression studies in squamous cell carcinoma of oropharynx and larynx has been reported [20]. Expression of galectin-3 was confined only to areas of high levels of keratinization in moderately/ highly differentiated carcinomas. No gal-3 expression was observed in the basal layers of cells studied in normal epithelium. The supra basal layers were positive in epidermis, epithelium of tongue and cornea and negative in the epithelium of palatine tonsil [20]. In another study of the squamous cell carcinoma and normal epithelium of tongue it has been reported that nuclear expression of galectin-3 markedly decreased during the progression from normal to cancer state while cytoplasmic expression increased [21]. Enhanced expression of galectin-3 in the cytoplasm has been reported to associate with reduced disease free survival of tongue cancer patients [17]. In the present study also the expression of galectins were confined to differentiated cell layers only in the normal and dysplastic epithelium. A similar observation has been reported in the squamous epithelium of oropharyngeal region and larynx suggesting its correlation with the extent of differentiation [20]. In malignancies the galectin-1 as well as galectin-3 was found to show higher expression in the differentiated areas uniformly in the entire squamous cell carcinomas studied. Higher expression patterns were observed in keratinising areas of squamous cell carcinomas as observed by Plazak. In adenocarcinomas, the expression for both the galectins were uniform in all the cases. Also the present study didn't observe any nuclear expression of galectins in any of the lesions analyzed. Expression of galectins particularly galectin-3 has been reported associate with progression of invasive tumors [22-25]. The molecular mechanism of this correlation has been proposed to be the interaction of galectin with Poly -N-actyl-lactosamines on laminin which may help in cellular invasion. The present study observed uniform expression of galectins in all the samples of invasive squamous cell carcinoma but in the preneoplastic lesions the expression was similar to that of normal samples except samples with CIN III, suggesting the malignant potential of this lesion. Here also, no difference was observed within the group of severe dysplasia so preselection by galectin expression also is not possible. The present study observed similar tissue binding patterns for galectin-3 as well as for galectin-1 and for the plant lectin with identical sugar specificity in the squamous epithelium of the uterine cervix. In squamous cell carcinomas, intraepithelial neoplasias and in normal epithelium the pattern of staining was more or less same for both of the galectins and SGL. But in normal mucus secreting endocervical cells and in adenocarcinomas the expression of both of the galectins was confined to the apical membrane and the lumen whereas uniform binding of SGL with moderate intensity was observed all over the cytoplasm. It may be because SGL may have the potential to recognize intracytoplasmic galactose moieties also. The galectins may be much more sensitive to secreted mucin, as it showed expression in the lumen of the glands as well as the apical areas of normal endocervical cells where secreted mucin is often found. Neither of the galectins nor the SGL may be capable of recognizing the changes in glycosylation pattern in early neoplastic events. However, in malignancies a higher expression of the galectins and SGL binding were observed even though they were also differentiation dependent. Hence the present study found no application for SGL or for Galectins in predicting the malignant potential of CIN and suggest these lectins as markers of differentiation, which may have some application in pathology and cytology to distinguish differentiated tumors from undifferentiated ones.

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